

# Genetic Linkage Map of the Eastern Oyster *Crassostrea virginica* Gmelin

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**Abstract.** Amplified fragment length polymorphisms (AFLPs), along with some microsatellite and Type I markers, were used for linkage analysis in *Crassostrea virginica* Gmelin, the eastern oyster. Seventeen AFLP primer combinations were selected for linkage analysis with two parents and their 81 progeny. The 17 primer combinations produced 396 polymorphic markers, and 282 of them were segregating in the two parents. Chi-square analysis indicated that 259 (91.8%) markers segregated in Mendelian ratio, while the other 23 (8.2%) showed significant ( $P < 0.05$ ) segregation distortion, primarily for homozygote deficiency and probably due to deleterious recessive genes. Moderately dense linkage maps were constructed using 158 and 133 segregating markers (including a few microsatellite and Type I markers) from male and female parents, respectively. The male framework map consisted of 114 markers in 12 linkage groups, covering 647 cM. The female map had 84 markers in 12 linkage groups with a length of 904 cM. The estimated genome length was 858 cM for the male map and 1296 cM for the female map. The observed genome coverage was 84% for the male and female map when all linked markers were considered. Genetic maps observed in this study are longer than the cytogenetic map, possibly because of low marker density.

## Introduction

The past decade has brought tremendous advances in genomics. Complete genome sequences are now available for many organisms including several eukaryotes such as *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Homo sapiens*. The list of completed genomes is rapidly

growing, and representative organisms of most taxa are now subjects of intensive genomic analysis. Mollusca, the second largest phylum of invertebrates, has received little attention. The number of available markers remains small and inadequate for genomic-wide mapping analysis, and no genetic linkage map has been published for any species. Understanding the genome of molluscs is important for comparative and evolutionary genomics, as well as for genetic improvement of cultured species.

*Crassostrea virginica* Gmelin, the eastern oyster, is one of the best-understood molluscs. It is a marine bivalve that occurs naturally along the Atlantic coast of North America (Galtsoff, 1964); it is easily cultured and widely available. It has a haploid number of 10 chromosomes (Longwell and Stiles, 1967), one of the lowest among molluscs, making it an ideal model mollusc for genetic and genomic analysis. The eastern oyster is also a species of economic significance, supporting major fishery and aquaculture industries in North America, so studies of its genome would be important for genetic improvement of this species. The development of genetic linkage maps is particularly useful for the mapping of quantitative trait loci (QTLs) and for marker-assisted selection (MAS) (Lander and Botstein, 1989; Cho *et al.*, 1994). Genetic linkage maps have been developed for almost all major aquaculturally important species, including tilapia, rainbow trout, catfish, oysters, and shrimps (Kocher *et al.*, 1998; Young *et al.*, 1998; Sakamoto *et al.*, 2000; Waldbieser *et al.*, 2001; Hubert *et al.*, 2002; Li and Guo, 2002; Moore *et al.*, 1999; Wilson *et al.*, 2002).

Two preliminary linkage maps were recently constructed for the Pacific oyster (*Crassostrea gigas*): one with microsatellites (Hubert *et al.*, 2002) and one with RAPD and AFLP markers (Li and Guo, 2002). However, details of the two maps have not been published. As for the eastern

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oyster, only a few microsatellites are available (Brown *et al.*, 2000), but more are being developed for population analysis and mapping (Reece *et al.*, 2002). Two linkage groups have been identified involving seven allozyme markers (Foltz, 1986).

While microsatellites are the most popular markers for genomic mapping, their development and application are slow and expensive. In oysters, many microsatellites suffer from surprisingly high levels of null-alleles, segregation distortion, and influence of repetitive elements (Launey and Hedgecock, 2001; Reece *et al.*, 2002; Gaffney, 2002). Amplified fragment length polymorphisms (AFLPs) are also popular markers for genome mapping, especially in "orphan" species where genetic resources are limited. AFLP markers are specific to restriction sites and thus reliable, and large numbers of polymorphisms can be developed quickly without prior knowledge of DNA sequence (Vos *et al.*, 1995). AFLPs have been developed and used for genome mapping in many aquatic animals, including catfish (Liu *et al.*, 1998, 1999a), rainbow trout (Young *et al.*, 1998), tilapia (Kocher *et al.*, 1998; Agresti *et al.*, 2000), medaka (Naruse *et al.*, 2000), Pacific oyster (Li and Guo, 2002), and shrimps (Moore *et al.*, 1999; Wilson *et al.*, 2002). We report here the construction of the first linkage maps for the eastern oyster with AFLP and a few microsatellite and Type I markers.

## Materials and Methods

### *Oysters and DNA extraction*

Eastern oysters used in this study were from a reference family (NEI-1) produced from the Rutgers NEH strain. The NEH strain was developed from Long Island Sound populations by long-term selective breeding since the early 1960s for resistance against MSX (a parasitic disease caused by *Haplosporidium nelsoni*) (Ford and Haskin, 1987) and recently for resistance against Dermo (a parasitic disease caused by *Perkinsus marinus*). The reference family was produced in 1998 by single-pair mating and was sampled in 1999 when the progeny were about one year old. Oyster tissues were kept in a  $-80^{\circ}\text{C}$  freezer before use. DNA was extracted from adductor muscle or mantle tissue from each animal using the CTAB phenol/chloroform protocol as described in Grewe *et al.* (1993). Two parents and four progeny were used for marker screening, and linkage analysis was based on 81 progeny.

### *AFLP markers*

AFLP analysis was conducted primarily according to Perkin-Elmer's (PE) AFLP plant mapping protocol, with some modifications. Adaptors, preselective primers, selective primers, and PCR reagents were purchased from PE. Restriction enzymes and T4 DNA ligase were purchased

from New England Biolabs (NEB). Genomic DNA ( $\sim 0.5\ \mu\text{g}$ ) was digested by restriction enzymes *EcoRI* and *MseI*, and ligated with relevant adaptors overnight at room temperature. Each reaction (11  $\mu\text{l}$ ) contained 5.5  $\mu\text{l}$  DNA, 1.0  $\mu\text{l}$  of  $10\times$  T4 DNA ligase buffer with EDTA, 1.0  $\mu\text{l}$  of 0.5 M NaCl, 0.5  $\mu\text{l}$  of 1.0 mg/ml BSA, 1.0  $\mu\text{l}$  each of *MseI* and *EcoRI* adaptors, and 1.0  $\mu\text{l}$  enzyme mix (0.1  $\mu\text{l}$  of  $10\times$  T4 DNA ligase buffer with EDTA, 0.1  $\mu\text{l}$  of 0.5 M NaCl, 0.05  $\mu\text{l}$  of 1.0 mg/ml BSA, 1.0 U *MseI*, 5.0 U *EcoRI*, 1.0 Weiss U T4 DNA ligase and proper amount of water).

Preselective primers complementary to each adaptor sequence only were used to amplify the restriction fragments created in the digestion-ligation step. Every 4  $\mu\text{l}$  of diluted (20-fold) digestion-ligation product was amplified in a 20- $\mu\text{l}$  reaction mixture containing 0.5  $\mu\text{l}$  of each *EcoRI* and *MseI* preselective primers, and 15  $\mu\text{l}$  PCR Core Mix. Preselective PCR was run at a temperature profile of one cycle of  $72^{\circ}\text{C}$  for 2 min, 25 cycles of  $94^{\circ}\text{C}$  for 25 s,  $56^{\circ}\text{C}$  for 30 s, and  $72^{\circ}\text{C}$  for 2 min, and one cycle of  $60^{\circ}\text{C}$  for 30 min. Products from preselective PCR were diluted 20-fold with Tris-EDTA buffer and used as templates for selective amplification. Pairs of selective primers, each containing two or three selective nucleotides at their 3' end, were used for selective PCR, with the *EcoRI* selective primer being fluorescein-labeled. The *EcoRI* and *MseI* selective primers were coded with letters and numbers, respectively (see Table 1). AFLP markers were named by adding the letter "F" (fragment) and three digits representing fragment size in base pairs to the end of the primer combination names. For example, marker F1F128 refers to a 128-bp fragment amplified from the primer combination of E-ACT [F] and M-CAA [1]. Amplifications were carried out in a 20- $\mu\text{l}$  reaction that was composed of 3.0  $\mu\text{l}$  diluted preselective product, 1.0  $\mu\text{l}$  *MseI* selective primer at 5.0  $\mu\text{M}$ , 1.0  $\mu\text{l}$  *EcoRI* selective primer at 1  $\mu\text{M}$ , and 15  $\mu\text{l}$  PCR Core Mix. Selective PCR was run with a touch-down profile: 10 cycles of  $94^{\circ}\text{C}$  for 20 s,  $66^{\circ}\text{C}$  for 30 s, and  $72^{\circ}\text{C}$  for 2 min, with a  $1^{\circ}\text{C}$  decrease in annealing temperature each cycle, followed by 20 cycles of amplification at  $94^{\circ}\text{C}$  for 20 s,  $56^{\circ}\text{C}$  for 30 s, and  $72^{\circ}\text{C}$  for 2 min.

Electrophoresis and data collection were carried out on an ABI 310 genetic analyzer (PE). After selective amplification, 1.0  $\mu\text{l}$  of PCR product was added to a 0.5-ml sample tube containing 12.0  $\mu\text{l}$  deionized formamide and 0.5  $\mu\text{l}$  GeneScan-500 size standard (PE). Samples were denatured at  $95^{\circ}\text{C}$  for 5 min, and then immediately cooled on ice for 5 min before being loaded onto the ABI 310 genetic analyzer. Electrophoresis parameters were set at injection for 10 s at 15 kv, running for 30 min at 13 kv and  $60^{\circ}\text{C}$ , with POP4 polymer. Data were collected using the GS STR POP4 A module in the Data Collection Software (ver. 1.0.2), and then analyzed with GeneScan Analysis software (ver. 3.1). Genotyper software was used to analyze and

score genotypes, and electrophoretic histograms were manually examined for genotyping errors.

#### Microsatellite markers

Ten microsatellite primer pairs were screened for polymorphism in parents. Seven of them were developed by Brown *et al.* (2000): *Cvi6*, *Cvi7*, *Cvi8*, *Cvi9*, *Cvi11*, *Cvi12*, and *Cvi13* (GenBank accession No. AF276247-AF276254). Three were developed in our lab, with one (*RU001*) polymorphic in the female parent. *RU001* is a (TA)<sub>28</sub> repeat with primer sequences of 5'-GGCTGCCAAATGAATAAATC-3'/5'-GAGTTTGGTCTCACACTTGAAATC-3', an annealing temperature of 54 °C, and an expected product size of 180. PCR reactions were performed in a 15- $\mu$ l volume containing 1 $\times$  PCR buffer, 2.5 mM of MgCl<sub>2</sub>, 0.2 mM of each dNTP, 0.5  $\mu$ M of each primer, 50 ng of genomic DNA, and 0.5 U of *Taq* polymerase (Promega, Madison, WI) using the following temperature profile: 94 °C for 2 min, then 30 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 15 s. Amplification products were resolved by electrophoresis on 10% acrylamide gel and stained with ethidium bromide.

#### Type I (EST) markers

cDNA sequences were obtained from suppression subtractive hybridization (SSH) libraries constructed with control and *Perkinsus marinus*-challenged eastern oysters (A. Tanguy *et al.*, Rutgers University, unpubl. data). Ten sequences were selected and screened for single-strand conformation polymorphisms (SSCPs). Two of them (*Rho* and *Vb65*) were polymorphic in at least one of the parents. The primer sequences are 5'-GGAATGGTGCTCTGTTTCACT-3'/5'-CCTATAATCCTGGTAGGCAACA-3' for *Rho*, 5'-ACATACAGCACCAAGAAAAAGC-3'/5'-AGAGATCACTATTTCCCTGCAC-3' for *Vb65*, and both with an annealing temperature of 52 °C. The amplified fragments were 391 bp for *Rho* and 380 bp for *Vb65*. PCR reactions were performed in 20  $\mu$ l containing 1 $\times$  PCR buffer, 2.0 mM of MgCl<sub>2</sub>, 0.2 mM of each dNTP, 0.25  $\mu$ M of each primer, 50 ng of genomic DNA, and 0.5 U of *Taq* polymerase (Promega, Madison, WI) using the following profile: 94 °C for 2 min, then 32 cycles of 94 °C for 45 s, 52 °C for 45 s, and 72 °C for 1 min. PCR fragments were denatured at 95 °C for 5 min and immediately chilled on ice. SSCP was separated on 8% acrylamide gel with 5% glycerol and visualized with silver-staining (Black and Duteau, 1997).

#### Data analysis and map construction

For AFLPs, primer combinations were evaluated based on the total number of total peaks produced, the number of

polymorphic (among two parents) peaks, and peak quality. Each peak was considered as a locus and scored as peak presence (*A/A*, *A/a*), or absence (*a/a*). Loci at which one parent was *A/a* and the other was *a/a* were selected for mapping analysis. Genotypes were coded as H for *A/a* and A for *a/a* in a backcross model. For microsatellite and Type I markers where more than two alleles were involved, one of the segregating alleles was selected and coded in the same way as AFLPs in a backcross model: H for present and A for absent. All segregating loci scored were checked with the chi-square test for goodness of fit to the 1:1 Mendelian ratio. Distorted markers were included in mapping analysis for possible identification of regions of distortion. Distorted markers with homozygote deficiency or homozygote excess were designated by adding a “-” or “+” to the end of the marker label. Linkage analysis was performed with MAPMAKER 3.0 (Lander *et al.*, 1987). Markers were grouped at a logarithm of odds (LOD) score of 3.0 and maximum distance of 38 cM first. Small groups ( $\leq 8$  markers) were ordered with multipoint exhaustive analysis using the “compare” command. Large groups were processed with the three-point analysis and “order” command. Additional markers were added by lowering the LOD score to  $\geq 2.0$  to obtain a framework map. Map order was verified with the “ripple” command. Some markers were clearly linked to specific groups but could not be placed on the maps, because they had more than one possible position (or were in different order) on the map. Typing errors were detected with the “error detection on” option, and map distances were computed in Kosambi function (Ott, 1999). Linkage groups were drawn with MAPCHART (Voorrips, 2001).

#### Marker distribution and genome coverage

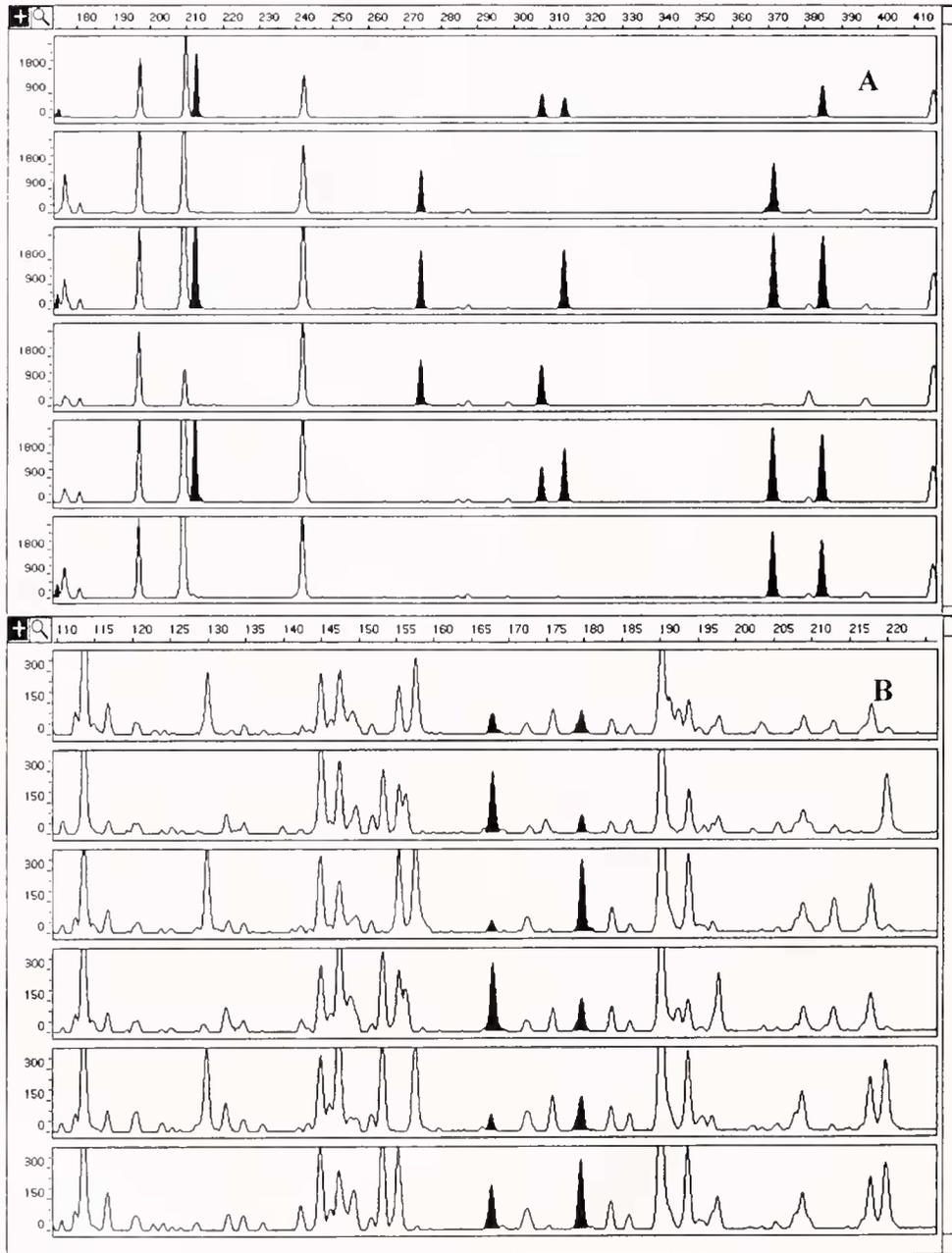
Marker distribution was analyzed by calculating the Pearson correlation coefficient between the number of markers in the linkage groups and the size of the linkage groups (SPSS ver. 8.0). Three methods were used to calculate the estimated genome length. First, we calculated the average marker spacing/interval (*s*) by dividing the total map length by the number of intervals (number of markers minus number of linkage groups). The estimated genome length ( $G_{e1}$ ) was determined by adding 2*s* to the length of each linkage group to account for chromosome ends (Fishman *et al.*, 2001). Secondly, an estimated genome length ( $G_{e2}$ ) was calculated by multiplying the length of each linkage group by  $(m + 1)/(m - 1)$ , where *m* is the number of framework markers in each group (Chakravarti *et al.*, 1991). Finally, we estimated genome length with a subset of well-spaced markers by  $G_{e3} = N(N - 1)X/K$ , where *N* is the number of markers, *X* is the maximum interval between two adjacent markers, and *K* is the number of marker pairs at an LOD score of 3.0 (Hulbert *et al.*, 1988; Chakravarti *et al.*,

1991). The average of the three estimates was used as the estimated genome length ( $G_e$ ) for the eastern oyster. Two observed genome lengths were calculated, one as the length of the framework map ( $G_{of}$ ), and the other as the total length considering all markers ( $G_{oa}$ ) (Cervera *et al.*, 2001). The observed genome coverages,  $C_{of}$  and  $C_{oa}$ , were determined by  $G_{of}/G_e$  and  $G_{oa}/G_e$ , respectively.

## Results

### AFLP markers

Fifty-six selective primer combinations were screened. AFLP profile or peaks generated by the AFLP protocol used were highly specific and reproducible when the selective amplification was successful (Fig. 1A). The majority of the



**Figure 1.** Representative AFLP histograms for two parents (top two panels) and four progeny (bottom four) of *Crassostrea virginica*. (A) Segregating peaks (highlighted) from primer combination E-ACT/M-CAG [F3]; peak presence and absence were scored as *Aa* and *aa*, respectively. (B) Co-dominant peaks (highlighted) from E-TG/M-CAG [03]: from top to bottom, genotypes were recorded as *Aa*, *AA*, *Aa*, *AA*, *Aa*, and *AA* for the highlighted peak on the left, and *Aa*, *Aa*, *AA*, *Aa*, *Aa*, and *AA* for the highlighted peak on the right.

peaks or fragments were between 60 and 450 bp. Fragments smaller than 60 bp were generally too crowded to score, and fragments bigger than 450 bp had low peak height or intensity. Most AFLPs are dominant markers, although co-dominance was apparent for some peaks (about seven loci), where *AA* and *Aa* genotypes were distinguishable by dosage or peak height (Fig. 1B). Dosage difference was clear for some peaks, but less certain for others. To be conservative, we did not include these co-dominant markers for mapping.

The number of polymorphic and all peaks produced by each of the 56 primer pairs are presented in Table 1. Except for one primer pair [O1], all primer pairs produced identifiable peaks, ranging from 17 to 106 peaks per pair; 2746 peaks were obtained from 55 primer pairs, averaging 50 peaks per pair. The average number of polymorphic peaks (among the two parents) was 747, or 13 per primer pair, which corresponds to an overall polymorphism of 27.2%.

The number of peaks produced among primer combinations varied considerably. Overall, the difference among the *MseI* primers was small, but among the *EcoRI* primers it was significant. For example, E-TA [M] produced 647 peaks in combination with eight *MseI* primers, while E-ACG [E] produced only 162 peaks (Table 1). The level of polymorphism produced by different primer combinations also varied considerably, ranging from 5.0% in E1 to 55.8% in F8. Overall, E-ACT [F] produced the highest level of polymorphism, 172 markers in combination with eight *MseI* primers or 38.5%.

On the basis of the number of peaks produced, the level of polymorphism, and peak quality, 31 primer combinations were considered as good, 11 as fair, and 14 as poor. The good primer combinations produced large numbers of well-separated peaks with little background. Primer combinations that produced few or poorly defined peaks or high background were considered poor. Seventeen good primer

combinations were selected for further evaluation and mapping analysis (printed in bold in Table 1). The 17 selected primer pairs produced 1056 peaks, and 396 of them (37.5%) were polymorphic in the reference family. Among the polymorphic markers, 298 were segregating in either the male or the female parent. Seven co-dominant markers (scored according to dosage difference) were not used for possible genotyping errors, and the remaining markers were scored qualitatively as peak present or absent. Nine markers were discarded because some progeny had unreliable or ambiguous genotypes. For the 282 polymorphic markers used, 153 (54.3%) were segregating through the male parent, and 129 (45.7%) through the female parent. Chi-square analysis indicated that 259 (91.8%) segregated according to the Mendelian ratio (1:1), and 23 (8.24%; 17 in male and 6 in female) showed significant segregation distortion ( $P < 0.05$ ). Among the 23 distorted loci, four (17.4%) were deficient in heterozygotes (*Aa*), and 19 (82.6%) were deficient in homozygotes (*aa*).

#### Microsatellite and Type I markers

Five of the ten microsatellites screened were polymorphic and segregating in at least one of the parents: *Cvi9*, *Cvi11*, *Cvi12*, *Cvi13*, and *RU001*. Among these five, *Cvi11* and *RU001* segregated in the female parent; *Cvi9* and *Cvi12* segregated in the male parent; and *Cvi13* segregated in both parents. Two of the 10 Type I markers (*Rho* and *Vb65*) were polymorphic when screened for SSCP. *Rho* was segregating in the male. *Rho* is a family of GTPases that regulate diverse cellular events including transcription, cell growth, development, and endocytosis and exocytosis (Leung *et al.*, 1999). *Vb65* is an unknown gene segregating in both parents. Chi-square analysis indicated that all microsatellite and Type I markers were in agreement with Mendelian ratios.

Table 1

Number of polymorphic and all peaks (before and after slashes, respectively) produced from 56 AFLP primer combinations in a family of *Crassostrea virginica* with polymorphic percentages in parenthesis

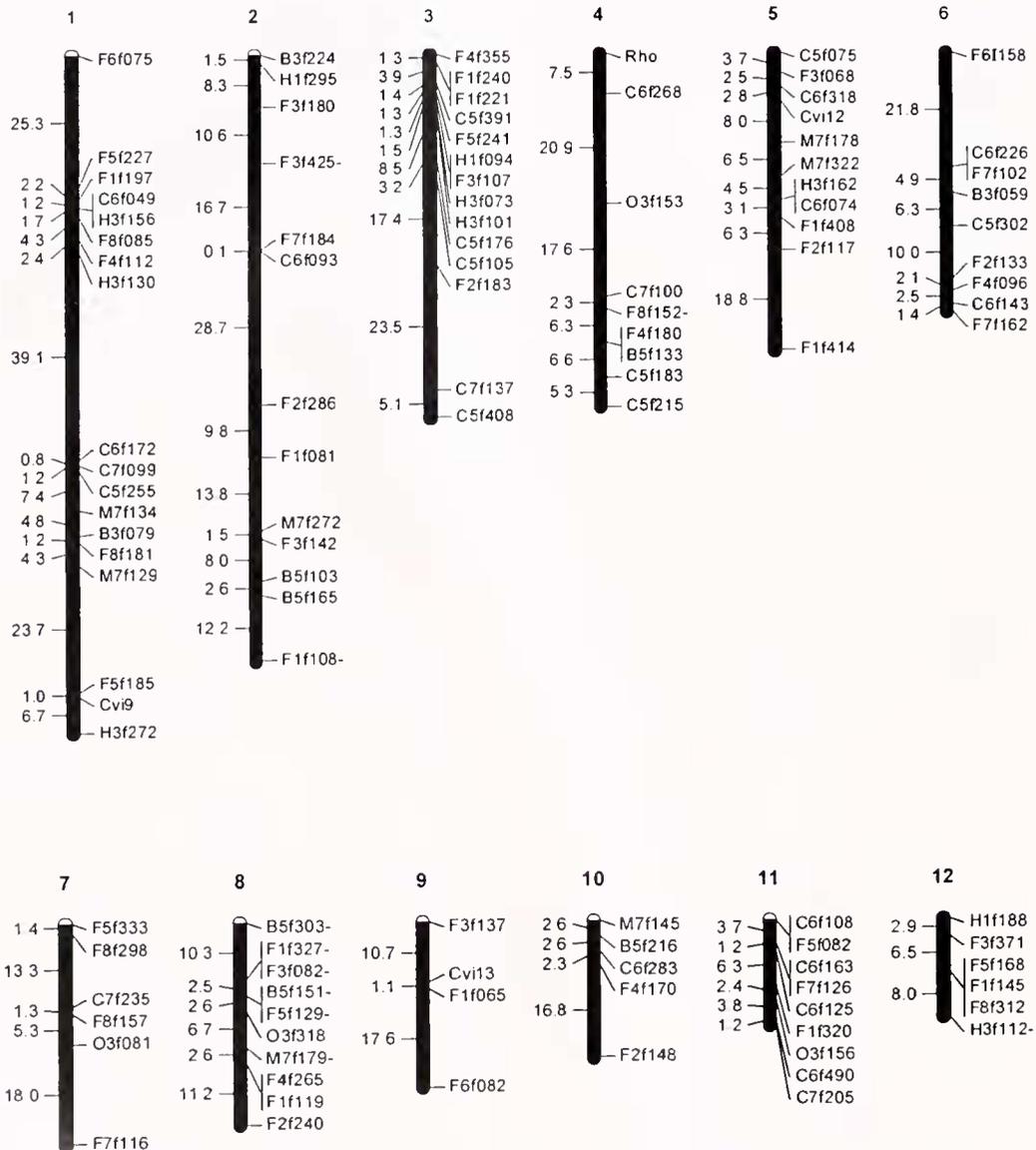
Primer	E-AAG [B]	E-ACA [C]	E-ACG [E]	E-ACT [F]	E-AGG [H]	E-TA [M]	E-TG [O]	Mean	Total
M-CAA [1]	4/42 (9.5)	11/51 (23.5)	1/20 (5.0)	<b>28/71 (39.4)</b>	<b>22/59 (37.3)</b>	7/69 (10.1)	Nodata	12/52 (20.8)	73/312
M-CAC [2]	2/26 (7.7)	9/35 (25.7)	3/19 (15.8)	<b>21/53 (39.6)</b>	5/37 (13.5)	10/73 (13.7)	12/49 (24.5)	9/42 (20.1)	62/292
M-CAG [3]	<b>20/49 (40.8)</b>	8/26 (30.7)	3/17 (17.6)	<b>26/57 (45.6)</b>	<b>21/53 (39.6)</b>	19/97 (19.6)	<b>13/54 (24.1)</b>	16/50 (31.2)	110/353
M-CAT [4]	11/50 (22.0)	9/42 (21.4)	8/25 (32.0)	<b>19/81 (23.5)</b>	7/32 (21.8)	21/106 (19.8)	9/42 (21.4)	12/54 (23.1)	84/378
M-CTA [5]	<b>21/66 (31.8)</b>	<b>33/85 (38.8)</b>	5/22 (22.7)	<b>18/46 (39.1)</b>	8/35 (22.8)	20/79 (25.3)	12/37 (32.4)	17/53 (30.5)	117/370
M-CTC [6]	12/37 (32.4)	<b>34/73 (46.6)</b>	4/17 (23.5)	<b>12/45 (26.7)</b>	9/55 (16.4)	13/62 (21.0)	8/32 (25.0)	13/46 (27.4)	92/321
M-CTG [7]	8/43 (18.6)	<b>36/72 (50.0)</b>	5/21 (23.8)	<b>19/50 (38.0)</b>	18/59 (30.5)	<b>24/90 (26.7)</b>	2/25 (8.0)	16/51 (28.0)	112/360
M-CTT [8]	15/57 (26.3)	31/96 (32.3)	7/21 (33.3)	<b>29/52 (55.8)</b>	4/30 (13.3)	7/71 (9.9)	4/33 (12.1)	14/51 (26.1)	97/360
Mean	12/46 (23.6)	21/60 (33.6)	5/20 (21.7)	22/57 (38.5)	12/45 (24.4)	15/81 (18.3)	9/39 (21.1)	13/50 (25.9)	
Total	93/370	171/480	36/162	172/455	94/360	121/647	60/272		747/2746

*EcoRI* primers (E-) are coded by [letters], and *MseI* primers (M-) are coded by [numbers]. Numbers in bolds are primer combinations used for linkage mapping in this study.

### Linkage maps and marker distribution

Two linkage maps were constructed; one for the male parent, using 158 markers; the other for the female parent, using 133 markers. The male framework map consisted of 114 markers in 12 linkage groups (Fig. 2). Twenty-one markers were not linked to the framework map, including 3 triplets, 5 doublets, and 2 unlinked markers (Table 2). Additionally, 23 unplaced markers were linked to the framework map but not placed, because they had uncertain or

conflicting positions on the map. The male map covered 647.4 cM in length, with a maximum interval of 39.1 cM and an average interval of 6.3 cM (Table 3). The length of the linkage groups ranged from 17.4 to 127.3 cM, and the number of markers varied from 4 to 18 per group. Microsatellite markers *Cvi9*, *Cvi12*, and *Cvi13* were mapped to Groups 1, 5, and 9, respectively. Type 1 marker *Rho* was placed on the distal region of Group 4, and *Vb65* was unlinked to any group. The 17 distorted AFLPs (suffixed with “-” or “+” in marker names for homozygote or hetero-



**Figure 2.** Genetic linkage maps of the male eastern oyster, *Crassostrea virginica*, with 114 markers and 647 cM. AFLP markers are labeled with two characters for the primer combination, followed by the letter “f” (for fragment) and a 3-digit fragment size in base pairs. Markers are indicated on the right; distances between markers (in Kosambi cM) are on the left. The suffix “-” designates distorted markers that are homozygote-deficient. Markers covered by thin vertical lines have no detectable distances among them. The 12 linkage groups were arranged according to map length.

**Table 2**

Number of markers used, mapped, unplaced, and unlinked in male and female *Crassostrea virginica*

Features	Male	Female
AFLP markers	153	129
Microsatellites	3	3
Type I markers	2	1
Total markers used	158	133
Mapped framework markers	114	84
Unplaced markers	23	12
Unlinked triplets	3	7
Unlinked doublets	5	4
Unlinked singles	2	8
Distorted AFLPs (%)	16 (10.5)	6 (4.7)

zygote deficiency, respectively) were distributed over five linkage groups, 1 triplet, 1 doublet, and 5 unplaced markers. Surprisingly, 6 of the 17 markers were located within a 22-cM region of Group 8, and all 6 markers were deficient for homozygotes. The six markers (*B5f303*, *F1f327*, *F3f082*, *B5f151*, *F5f129*, and *M7f179*) were not produced from a single primer combination, so the segregation distortion could not arise from anomalous amplification of these markers.

The female framework map consisted 84 markers in 12 linkage groups (Fig. 3). Not included in the framework map were 37 unlinked markers, including 7 triplets, 4 doublets, and 8 singles, and 12 linked but unplaced markers (Table 2). The length of the female map was 904.3 cM, the maximum interval was 35.2 cM, and the average interval was 12.6 cM. The length of the linkage groups ranged from 17.5 to 167.3 cM, and the number of markers varied from 4 to 15 per group (Table 3). Microsatellite markers *Cvi11*, *Cvi13*, and *RU001* were assigned to Groups 7, 2, and 6, respectively; Type I marker *Vb65* was linked to two markers outside the framework map. Four of the six distorted markers were placed on the framework map with each in a different linkage group. The microsatellite marker *Cvi13*, which was the only marker segregating in both male and female, was mapped to Group 9 of the male map and Group 2 of the female map, indicating that the two groups are the same chromosome.

Overall, AFLP markers were randomly distributed in the linkage maps, as indicated by the significant ( $P < 0.01$ ) correlation between the number of markers in the linkage groups and the size (length) of the linkage groups for both female and male maps. Some small clusters and gaps were noticeable in both maps. Clustering was more apparent in the male map than in the female map.

#### Genome length and genome coverage

Genome length, which was estimated by three methods, was similar, ranging from 799.8 to 958.5 cM for the male,

and from 1205.5 to 1489.3 cM for the female (Table 4). The average of the three estimates was used as the expected genome length for *C. virginica*: 858.0 cM for the male, and 1295.9 cM for the female. On the basis of the expected genome length, genome coverage for the male and female framework maps was 75.5% and 69.8%, respectively. When all linked markers (framework plus doublets and triplets) were considered, the observed genome length became 718.4 cM for male and 1091.6 cM for female maps, corresponding to 83.7% and 84.2% coverage over the respective genomes. Clearly, the female map was longer than the male map.

## Discussion

### Marker evaluation, distribution, and segregation distortion

This study demonstrates that, in *Crassostrea virginica*, the eastern oyster, as in other organisms (Liu *et al.*, 1998; Kocher *et al.*, 1998; Agresti *et al.*, 2000; Wilson *et al.*, 2002), AFLPs are well suited for linkage mapping. AFLPs are highly specific, reproducible, and relatively simple to

**Table 3**

Length number of markers, average spacing, and largest intervals of linkage groups in female and male map of *Crassostrea virginica*

Linkage group	Length (cM)	Number of markers	Average interval (cM)	Largest interval (cM)	
Male	1	127.3	18	7.5	39.1
	2	113.8	13	9.5	28.7
	3	68.4	14	5.3	23.5
	4	66.5	9	8.3	20.9
	5	56.2	11	5.6	18.8
	6	49.0	9	6.1	21.8
	7	39.3	6	7.9	18.0
	8	35.9	10	4.0	11.2
	9	30.7	4	10.2	17.7
	10	24.3	5	6.1	16.8
	11	18.6	9	2.3	6.3
	12	17.4	6	3.5	6.5
Total	647.4	114	6.3 <sup>a</sup>		
Female	1	167.3	15	12.0	35.2
	2	116.6	9	14.6	34.7
	3	114.9	8	16.4	24.8
	4	111.1	10	12.3	33.1
	5	92.6	7	15.4	24.5
	6	72.8	6	14.6	25.7
	7	68.3	5	17.1	34.5
	8	62.5	6	12.5	28.8
	9	30.6	4	10.2	16.8
	10	26.9	4	9.0	25.3
	11	23.2	5	5.8	11.4
	12	17.5	5	4.4	7.6
Total	904.3	84	12.6 <sup>a</sup>		

<sup>a</sup> Average of all linked markers. Map distance (cM) is in Kosambi units.



**Figure 3.** Genetic linkage maps of the female eastern oyster, *Crassostrea virginica*, with 84 markers and 904 cM. AFLP markers are labeled with two characters for the primer combination, followed by the letter "f" (for fragment) and a 3-digit fragment size in base pairs. Markers are indicated on the right; distances between markers (in Kosambi cM) are on the left. The suffix "-" designates distorted markers that are homozygote-deficient. Markers covered by thin vertical lines have no detectable distances among them. The 12 linkage groups were arranged according to map length.

develop. The overall level of polymorphism—27.2% in a reference family with two parents originating from the same population—is reasonably high and adequate for mapping analysis. By selecting the best selective primer combinations, the number of polymorphic markers can be further increased to about 37.5%, or 23 per primer pair for the 17 selected primer pairs in this study. The proportion of polymorphic loci observed in this study is similar to the 42% observed from 12 selected primer combinations in tilapia

(Kocher *et al.*, 1998), the 39.1% for 64 primer combinations in catfish (Liu *et al.*, 1998), and the 40% for 35 primer pairs in silkworm (Tan *et al.*, 2001). The use of inter-strain crosses in the eastern oyster may further increase the polymorphism of AFLP markers and mapping efficiency. This study should encourage the use of AFLP markers in other molluscs, where genome mapping has so far been prevented primarily by the lack of molecular markers.

As expected, the majority of AFLP markers followed

Table 4

Map length and genome coverage for *Crassostrea virginica*

Map length (cM)	Male	Female
Observed length		
$G_{of}$	647.4	904.3
$G_{oa}$	718.4	1091.6
Estimated length		
$G_{e1}$	799.8	1205.5
$G_{e2}$	815.6	1192.8
$G_{e3}$	958.5	1489.3
Average $G_e$	858.0	1295.9
Genome coverage (%)		
$C_{of}$	75.5	69.8
$C_{oa}$	83.7	84.2

Mendelian segregation ratios. The level of segregation distortion (8.2% of loci) observed in this study is low and within the range observed in most other species, from none in a shrimp (Moore *et al.*, 1999) and catfish (Liu *et al.*, 1998), to 8% in tilapia (Kocher *et al.*, 1998) and 13.3% in rainbow trout (Young *et al.*, 1998). The silkworm, where 54% of the loci showed segregation distortion in a backcross (Tan *et al.*, 2001) is an apparent exception. Segregation distortion has been reported in the eastern oyster with other types of genetic markers, including in 7% of anonymous single-copy nuclear DNA polymorphism loci (Hu and Foltz, 1996) and in 11.5% of microsatellite loci (Reece *et al.*, 2002). In another study with allozyme markers, segregation distortion was found in 48.3% of cases tested (Foltz, 1986), although there were problems with cross contamination. In the Pacific oyster, segregation distortion has been observed in 31% of allozyme loci (McGoldrick and Hedgecock, 1997), 20.9% of microsatellites (Launey and Hedgecock, 2001), and 27% of AFLP markers (Li and Guo, 2002). Segregation distortion seems to be less frequent in the eastern oyster than in the Pacific oyster, *Crassostrea gigas* which may reflect true differences between the two genomes. Besides oysters, segregation distortion has been also reported in several other bivalves (Beaumont *et al.*, 1983; Gaffney and Scott, 1984; also see review in Wilkins, 1976).

In the Pacific oyster, the high level of segregation distortion is primarily due to a deficiency in identical-by-descent homozygotes caused by high genetic load (Launey and Hedgecock, 2001). Homozygote-deficiency accounted for 76% of all segregation distortions in this study, which also suggests selection against deleterious recessive mutations. Different levels of segregation distortion may reflect differences in the lineage or genetic load of the populations or crosses studied. Families used in the present study and that of Hu and Foltz (1996), where the lowest segregation distortion (7% and 8.2%) was found, came from Rutgers stocks that have been subjected to long-term selection (mass selection over 10 generations for disease resistance). This

long-term selective breeding may have eliminated some deleterious recessive genes. The mapping of distorted markers may help us understand the distribution of deleterious recessive genes in the genome. In the male map, 6 of the 17 distorted loci are closely linked, and all 6 loci are homozygote-deficient, indicating that they are linked to an unknown gene with a recessive deleterious allele. We named this gene *Dru01* (deleterious recessive unknown), and on the basis of the level of distortion and distance among the six markers involved, tentatively placed it in a distal region of Group 8 between markers *B5f303* and *F1f327*. It would be interesting to determine when *Dru01* is expressed (before or after metamorphosis) and how prevalent the deleterious allele is.

AFLP markers may distribute randomly in some species (Castiglioni *et al.*, 1999; Remington *et al.*, 1999; Cervera *et al.*, 2001), but form clusters in others (Young *et al.*, 1998; Waldbieser *et al.*, 2001; Sakamoto *et al.*, 2000). The distribution of AFLP markers in the eastern oyster is mostly random, and the level of clustering observed is considerably lower than that in rainbow trout (Young *et al.*, 1998). Clustering in rainbow trout and some plants (Nandi *et al.*, 1997; Castiglioni *et al.*, 1998) occurred in centromere regions. Oyster chromosomes are mostly metacentric, and our maps show that large intervals are often located in the central regions of most large linkage groups in both female and male maps (Figs. 2 and 3); these regions may correspond to recombinant hot regions near centromeres. It has been suggested that crossover hot regions exist in the marine bivalve *Mulinia lateralis* and occur near the centromere region (Guo and Allen, 1996). Because *EcoRI* and *MseI* restriction sites are relatively AT-rich, clustering may be a reflection of variation in GC content among chromosomal regions. In maize, Castiglioni *et al.* (1999) reported that AFLP markers generated by *PstI/MseI* primer combinations were more evenly distributed than those by *EcoRI/MseI* primers, because the restriction site of *PstI* is relatively GC-rich.

#### Linkage map, map length, and genome coverage

This study provides the first estimated genetic map length in the eastern oyster: 858.0 cM for the male map and 1295.9 cM for the female map. Sex differences in crossover frequencies and map length are commonly observed, with the male map often shorter than the female map in vertebrates (Dib *et al.*, 1996; Dietrich *et al.*, 1996; Sakamoto *et al.*, 2000; Singer *et al.*, 2002). Similar differences in map distances between males and females have been noted in the Pacific oyster (D. Hedgecock, Bodega Marine Laboratory, UC Davis, pers. comm.; Li and Guo, 2002). The estimated length for both male and female maps, however, is considerably longer than the expected length based on chiasma data. Cytogenetic observation of chromosome pairing suggests that there is about 1.1–1.3 chiasmata per chromosome

in eggs and 1.0–1.1 chiasmata per chromosome in primary spermatocytes of the eastern oyster (Guo *et al.*, unpubl. data), which corresponds to a theoretical length of 550–650 cM for the female map, and 500–550 cM for the male map. The estimated genome length based on our linkage data is about 43% and 147% longer than that of the cytogenetic map for the male and female, respectively.

The discrepancy between the cytogenetic and genetic map length may be caused by several factors. First, the number of chiasmata observed at metaphase may be an underestimate of crossover events. It has been suggested that closely formed crossover events can lead to chiasma reduction (Nilsson *et al.*, 1993; Nilsson, 1994). But chiasma reduction has not been accepted as a prevailing meiotic event, and cytogenetic length is close to genetic length in most well-studied model species (Morton, 1991). Chiasma frequency may underestimate cytogenetic map length to some extent; however, it is unlikely to account for the 145% difference in length between the cytogenetic and linkage maps. Secondly, map length may be inflated by the linkage mapping program MAPMAKER (Sybenga, 1996). In yellow monkeyflower, MAPMAKER overestimated map length by 20% (Fishman *et al.*, 2001), which may have happened to our maps; however, it is inconceivable that MAPMAKER could cause 147% inflation. Thirdly, typing errors are known to inflate map length. We constructed maps without error detection first, but the final maps presented here were constructed with the "error detection on" option in MAPMAKER. Error detection resulted in a 20% reduction in length for the male map and a 9% reduction for the female map. Error detection has led to different levels of length reduction in other organisms: 4.3% in the silkworm (Tan *et al.*, 2001), 14% in tilapia (Koehler *et al.*, 1998), and 40.3% in barley (Castiglioni *et al.*, 1998). Finally, the genetic map may be inflated by low marker density. Genetic maps usually shorten with increased marker density. In medaka, for example, 170 markers produced a 2480-cM map in 29 linkage groups (Wada *et al.*, 1995), whereas 663 markers produced a high-density map of just 1354.5 cM in 24 linkage groups (Naruse *et al.*, 2000). Similarly, in the silkworm, 356 AFLP-markers produced a map of 6512 cM (Tan *et al.*, 2001), while 1018 RAPD markers produced a map of only 2000 cM (Yasukochi, 1998). In rice, a map constructed with 762 markers measured 4026.3 cM (Causse *et al.*, 1994), while the map built with 2275 markers measured only 1521.6 cM (Harushima *et al.*, 1998). Low marker density is likely the primary cause for the longer than expected genetic length observed in this study, although other factors may have contributed to the overall increase in map length. We expect that, as marker density increases, the genetic maps will decrease in length and become comparable to the cytogenetic map.

The linkage maps presented here are the first genetic maps for the eastern oyster. They are similar in marker

density to a 98-marker, 880-cM microsatellite map (Hubert *et al.*, 2002), and a 104-marker, 1051-cM AFLP map (Li and Guo, 2002) of the Pacific oyster. The eastern oyster maps are obviously incomplete, as indicated by genome coverage and the presence of large gaps. The eastern oyster has a haploid number of 10 chromosomes (Longwell and Stiles, 1967), and the male and female linkage maps both had 12 linkage groups. The small linkage groups, as well as some triplets and doublets, may join other groups as marker density increases and gaps are filled. Despite the presence of gaps, the maps provide reasonably good coverage of the eastern oyster's genome: 83.7% for the male map and 84.2% for the female map when all linked markers are considered. With an average interval of 6.3–12.6 cM, these maps provide a basic framework for gene and QTL mapping in the eastern oyster, though marker density should be increased and more microsatellite and Type I markers should be added.

One concern with the AFLP markers is the transferability among populations and laboratories. AFLPs are rather consistent among families and crosses (Moore *et al.*, 1999; Wilson *et al.*, 2002; Roupe van der Voort *et al.*, 1997). Transferability among laboratories may depend on the scoring systems used. Fortunately, poor transferability can be compensated for by the ease of developing a large set of AFLP markers in any population of interest, thus limiting the need for transfer. In some cases, the interest is in a particular stock, and AFLP markers can be developed specifically for mapping and selection in that stock. There is no question that microsatellites, because of their high levels of polymorphism and co-dominance, are better markers for linkage mapping than are AFLPs. They are also more expensive to develop and to use. The best strategy could be to use AFLPs in combination with microsatellites (Koehler *et al.*, 1998; Young *et al.*, 1998; Agresti *et al.*, 2000). Microsatellites can provide a backbone for the linkage map and afford some transferability, while AFLPs would saturate the map and fill gaps with a large number of markers. A large number of oyster ESTs is becoming available (Jenny *et al.*, 2002) and can be added to maps. A high-density AFLP/microsatellite map can facilitate rapid mapping of EST/Type I markers and eventually comparative mapping.

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